2012

Boronic Acid Macroligands for Glycomics Applications

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BORONIC ACID MACROLIGANDS FOR GLYCOMICS APPLICATIONS

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Bachelor of Pharmacy

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MAY 2010

Submitted in partial fulfillment of requirements for the degree of

MASTER OF SCIENCE IN CHEMISTRY

At

CLEVELAND STATE UNIVERSITY

August 2012

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ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. Xue-Long Sun, for providing such interesting project to work on, giving me an opportunity to carry on this research in his lab, and also for his help, guidance, and motivation throughout all these years that helped me in the development of my scientific knowledge all the times of this research and writing of this thesis.

I would like to thank my committee members, Dr. Aimin Zhou, Dr. Bin Su, for their support and assistance throughout my academic career and my research work.

I am very grateful to Satya Nandana Narla who played a key role in this project and also other lab mates Pratima Vabbilisetty, Jayasri Kakarla, Dr. Yong Ma, Dr.Jacob J Weingart, Dr.Hailong Zhang, Dr. Rui Jiang, Lin Wang and Valentinas Gruzdys for their assistance through all the phases of my thesis study.

I would like to thank my parents and my grandparents whose constant support, love, and inspiration gave me the confidence and strength to accomplish my goals. Finally I would like to thank my friends and roommates Sruthi, Anuhya, Neelima, Ramya, Bavita, Bhargavi, and Prathyusha.

BORONIC ACID MACROLIGANDS FOR GLYCOMICS APPLICATIONS

POORNIMA PINNAMANENI

ABSTRACT

Cell surface carbohydrates existing as glycoproteins and glycolipids represent the first information about the cell with the outside world and are closely involved in various biological processes such as cell communication, and the molecular recognition and cell targeting. However, the mechanisms of most of the processes at the molecular level are still unclear. It is therefore very important to develop specific carbohydrate-binding molecules for a fast, efficient, sensitive and accurate analysis of complex carbohydrates structures and functions, known as glycomics. In addition, certain carbohydrate-binding molecules can be used in medical applications such as biomarkers for diseases diagnosis and targeted drug delivery applications. In this thesis study, I conducted synthesis, characterization and glyco-capturing capacity investigation of bovine serum albumin (BSA)-boronic acid (BA) conjugates as lectin mimetics. The BSA-BA conjugates were synthesized by amidation of carboxylic acid groups in BSA with aminophenyl boronic acid in the presence of EDC, and were characterized by Alizarin Red S (ARS) assay and SDS-PAGE gel. The BSA-BA conjugates were immobilized onto maleimide-functionalized silica beads and their sugar capturing capacity and specificity were confirmed by Alizarin Red displacement assay. As a result, the interactions between the sugars and SB-BSA-BA was in the order of

Fructose > Galactose > Mannose > Fucose > Lactose > Sialic Acid > GlcNAc > Glucose at pH 7.4 and Fructose > Fucose > Lactose > Galactose > Mannose > GlcNAc > Sialic Acid > Glucose at pH 8.3. Further, SPR analysis of BSA-BA-based glyco-capturing test was conducted by immobilizing BSA-BA onto SPR gold chip. As a result, the interactions between the sugars and BSA-BA on the gold chip were in the order of Lactose > Mannose > Glucose > Fucose > Sialic acid > Fructose > GlcNAc > Galactose at pH7.4 and Glucose > Galactose > Mannose > GlcNAc > Fructose > Fucose > Sialic acid > Lactose at pH8.3. Overall, I have developed a BSA-BA-based lectin mimetics for glycocapturing applications. These lectin mimetics will provide an important tool for glycomics and glycoproteomics research and applications.

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LIST OF ABBREVATIONS

ARS…… Alizarin Red S

BA……Boronic Acid

BSA…….Bovine Serum Albumin

EDC…… (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) HCl

SB…….. Silica Beads

SDS PAGE…… Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPR……....Surface Plasmon Resonance

CHAPTER I

INTRODUCTION

1.1 Introduction

Carbohydrates are one of the most important biomolecules in the cell that mostly exists as glycoproteins and glycolipids as information carrying molecules. They act as intermediates of biological communication and are involved in a wide range of functions such as cell growth, motility, cellular and molecular targeting, cell-cell interactions, morphology, cell recognition**,** they are also involved in the signaling pathway that determines the temporal and spatial identity of the cell (Figure 1.1). Nature use carbohydrates as an on-off switch to regulate biological events **(1)**. They have the ability to embody a cell-surface code for intercellular interactions. Complex carbohydrates coat the cell surface that helps in cell-cell recognition. These also help to interact with the sugars of the opposite cells, which results in adhesion of two cells **(2)**. They are also involved in host – pathogen interactions, bind to various compounds that enter the cell. However, the binding mechanism for most of the processes is still unclear. Indeed, the development of ligands that binds tightly and selectively to carbohydrates at molecular level is very difficult. This difficulty is explained by the conformational flexibility of the connections between various units of carbohydrates and the very

limited range of functional groups to interact. The detection and direct analysis of the carbohydrates is very difficult due to their characteristics such as lack of chromophores or fluorophores and general electro-neutrality. Hence, development of carbohydrate-binding molecules for identification and purification is necessary. Initial investigations reveal their usefulness in chromatographic applications for the separation of oligosaccharides. Further investigations proven the utility of such engineered modules for the assessment of carbohydrate composition not only of plant tissue sections but also of biotechnological processes designed to modify pulp fibers. They will thereby have an important impact on carbohydrate research in the future.

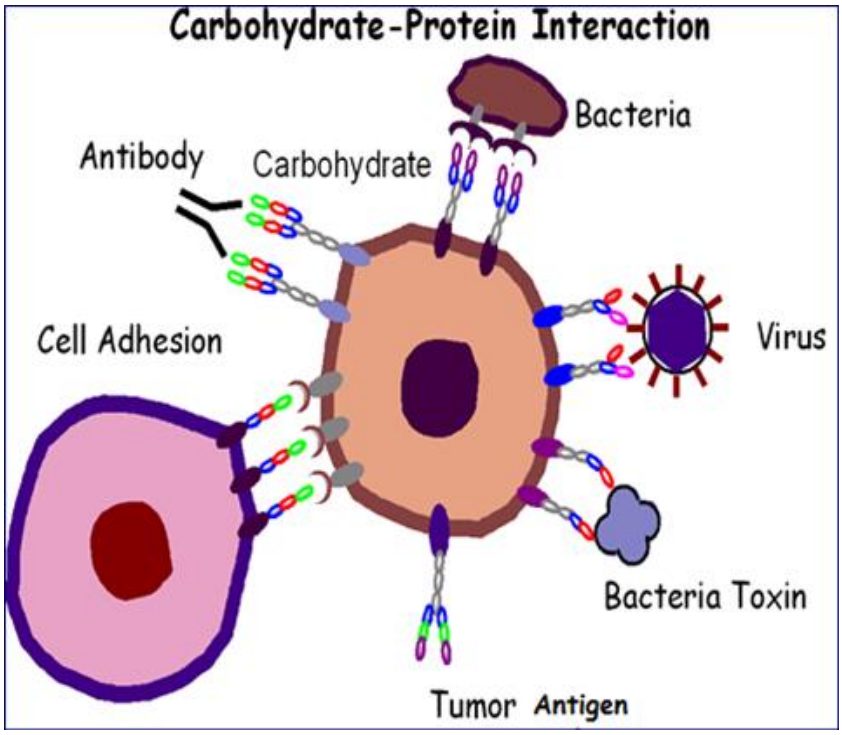


Fig 1.1: Cell surface carbohydrate-protein interactions in physiological and pathological processes.

1.2 Glycomics

Glycomics is the comprehensive study of the glycans in any form either as monosaccharide, complex glycans, glycoproteins or as glycolipids. Glycomics explores the role of carbohydrates in biological processes such as carbohydrate-carbohydrate, carbohydrate-protein, and carbohydrate-nucleic acid interactions. Glycans have important role in drug development as either glycoprotein therapeutics, or as saccharide-based therapeutics. Glycoproteins structure constitute of complex glycans, which can modulate the stability, half-life and activity of biotechnologically derived proteins. Glycans are used as drugs either in combination with proteins or alone as formulation of the glycans for drug delivery is easy, they are small and intrinsically more stable than protein based drugs, and are highly specific **(3)**. Glycosylation is the most common post translational method for altering the degree of branching and levels of glycans. The glycosylation machinery is highly sensitive to the biochemical environment. New technologies to study the complex glycans glycosylation have to be developed. Mass spectrometry-based glycomics provides the platform to monitor several diseases simultaneously by mass mapping strategies. This is done by obtaining compositions of the glycans **(4)**. Capillary electrophoretic **(5)** technique is also used for analyzing the glycan structures in minute amounts. These techniques not only provide information about the glycan structure and function but also differentiate the closely related glycans. For further structural characterization of glycans analytical techniques with analytical measurements like date integrations using bioinformatics were used. Instruments which are used for this purpose are capillary liquid chromatography, LC-NMR, etc.

The basic information about the mammalian glycoproteins is collected using a combined procedure of lectin affinity technology called “glyco-catch” and “2-dimensional liquid chromatography/mass spectroscopy/mass spectroscopy (2D-LC/MS/MS)”. However, MS shows difficulty in discrimination of number of structural isomers, such as enantiomers, diastereomers, etc. To overcome this lectins are used by Frontal affinity Chromatography (FAC) and also by Lectin arrays. Frontal affinity chromatography determines the dissociation constants between same number of lectins and oligosaccharides whereas in lectin arrays coincidental detection of multiple lectin-oligosaccharide interactions can be observed, which cannot be done by FAC **(6)**. However further studies are required to get the complete knowledge about the glycomics which is useful in new drug development and also in disease markers for many disease like cancers, angiogenesis, tissue repair etc.

1.3 Popular methods for isolation and identification of the carbohydrates and glycoconjugates

1.3.1 Lectin-Carbohydrate binding

Carbohydrates at molecular levels are recognized by proteins. The most intensively studied class of carbohydrate-binding proteins is lectins. Lectins are the naturally occurring molecules are found in animals, plants, bacteria and viruses. They bind reversibly to mono and oligosaccharides with strong affinities. They play a key role in the control of various normal and pathological processes in living organisms. Carbohydrate-Lectin interaction is a controlling step in myriad human disease state **(7)**. However, the mechanism of carbohydrate binding by

lectins is still unclear. They are devoid of catalytic activity and are not products of immune response in contrast with antibodies. The role of water in natural carbohydrate binding is still not known **(8)**. As most of the lectins are from natural sources there can be some variability in their binding affinities dependent on purification.

1.3.2 Hydrazide chemistry

When compared with lectins affinity approach hydrazide chemistry has high affinity towards glycoprotein's. The enrichment of the glycoproteins using hydrazide chemistry is based on covalent reaction **(9)**. In hydrazide chemistry first the *cis*-diol groups of the carbohydrates are converted to aldehyde groups by periodic oxidation, followed by coupling of aldehyde groups with hydrazide resin. It has more specificity to glycoproteins when compared with the lectin affinity. The main drawback is oxidation step which increases time and complexity whereas lectin affinity is simple and flexible. The nonglycosylated peptides are more analyzed (almost double the amount of glycosylated peptides) when compared to glycosylated peptides **(10)**.

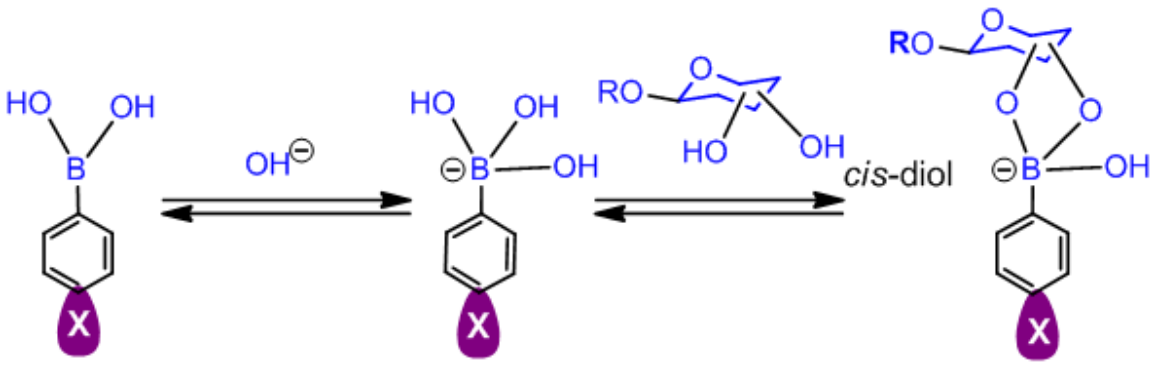
1.3.3 Hydrophilic interaction liquid Chromatography

Hydrophilic interaction liquid chromatography is also used for the enrichment of glycoproteins. In this a hydrophilic stationary phase is used along with an organic mobile phase and the elution is done by increasing the water concentration **(11)**. However, some glycoproteins have strong hydrophobic nature while some non-

glycoproteins have strong hydrophilic nature which may cause to produce false negative and false positive results.

1.4 Boronic acids as ligands for *cis*-diol

The interaction between borate and *cis*-diol had been employed as a tool in analysis of carbohydrates for many years. Borate ions in the boronic acid forms the reversible complexes with the diols in aqueous solution, this would affect the retention factor for the saccharides. These have the capacity to function as saccharides receptors in the aqueous solution they form the cyclic boronic esters with the carbohydrates under equilibrium conditions via reversible covalent interactions (Scheme 1). These interactions help to identify, separate and purify the monosaccharides, glycoprotein's also any number of carbohydrate-containing species such as, DNA, RNA. Borate/cis-diol interactions have also been used in molecular imprinting. Lectins binding to carbohydrates are used in many processes such as cell-cell interaction, adhesions of bacteria to animal tissues, etc. In the recent years many efforts were made to develop water soluble copolymers containing phenylboronic acid, to study their lectin like interactions with the cells. The binding of the carbohydrates with the boronic acids may differ due to various conditions like pH of the solutions in which the reaction is taking place, or due to multipoint chelation of the carbohydrate residues with the boronic acids **(12)**.



Scheme 1: Boronic acid as ligand for *cis*-diol

1.4.1 Boronic acid-containing macromolecules for carbohydrate characterization

In one of the earlier studies by C. Cannizzo **(13)** *et al*. reported boronic acid-functionalized nanoparticles as re-usable optical nanosensor for carbohydrates. First, reactive nanoparticles *NL-CH_2X* are prepared using 2, 2-dimethoxy-2-phenylacetophenone (DMPA) as initiator and dodecyl trimethylammonium bromide (DTAB) as surfactant, then boronic acid functionalized nanoparticles *NL-B $(OH)_2$* were prepared using 3-aminophenyl boronic acid monohydrate and sodium hydroxide. Sugar binding affinity is studied by the interactions of nanoparticles with fructose. It has shown the selectivity for the diols and also the phenylboronic acid grafted on the nanoparticles retains its binding affinity and also the nanoparticles are re-usable by dialysis process.

Boronic acids are also known to bind the glycoproteins and glycolipids. In one of the earlier studies Yawei Xu **(14)** *et al.* reported boronic acid functionalized Mesoporous silica for enrichment of the glycoprotein's. First, aminophenylboronic acid bonded GLYMO (GA) was prepared, to which mesoporous silica FDU-12

was added to form Mesoporous Di-Boronic Acid Functionalized FDU-12(FDU-12-GA). Enrichment of various glycoproteins from HRP, asialofetuin, fetuin, invertase is done and it is shown to have increased specificity by 83.5% and also the limit of detection of glycoprotein's have also been dramatically enhanced. The glycoprotein specificity and enrichment is not much affected even in the presence of high bound nonglycopeptides. H Lu **(15)** et al. further continued the work by Y Xu et al. and synthesized boronic acid functionalized core-satellite composite nanoparticles for advanced enrichment of glycoproteins and glycopeptides. The gold nanoparticles were synthesized using the previous literature by Y Xu et al. then boronic acid functionalized composite nanoparticles were synthesized by using 11-mercaptoundecanol (MUD) which servers as antenna, this helps to extend the functional groups as possible as it could in the solution. For the enrichment of the glycoproteins they have used a minute amount of the sample i.e., 0.1ng/µL. these composite nanoparticles identified the N-glycosylated proteins in a real biological sample and found to be more effective when compared with the commercially available SiMAG-boronic acid. In another study by Qi *et al.* **(16)** synthesis of mercaptophenylboronic acid-functionalized core-shell structure Fe_3O_4@C@Au magnetic microspheres for selective enrichment of glycopeptides and glycoproteins was reported. Fe_3O_4@C magnetic microspheres were synthesized using two-step reactions including solvothermal and hydrothermal reaction. Magnetic microspheres attain negative charge using glucose. Then poly (diallydimethylammonium chloride) (PDDA) was deposited onto the surface of the magnetic microspheres by electrostatic adsorption. The presence of a layer of positively charged PDDA on the surface of magnetic

microspheres ensures the efficient adsorption of the gold nanoparticles. Finally Fe_3O_4@C@Au magnetic nanoparticles were obtained by self-assembly approach. These magnetic nanoparticles were modified with 4-mercaptophenylboronic acid. Synthesized boronic acid functionalized nanoparticles were tested for capturing and enrichment of RNase B, myoglobin and tryptic peptide solution from horseradish peroxidase, results were analyzed using mass spectrometry. The results showed that synthesized boronic acid functionalized Fe3O4@C@Au nanoparticles shows specific enrichment for glycoprotein and also selectivity.

The diols binding with the boronic acids is also affected with the pH, pK_a, and binding constants. In the studies by J. Yan **(12)** *et al.* reported the substitution of the different functional groups in the phenylboronic acid may also affect the binding affinity towards the diols. They reported that the facts that lower the pK_a value higher binding affinity are not always true and many other factors also affect the binding affinity. Also as the pH of the solution increases the binding affinity also increases. At higher pH the boronic acid is known to acquire an additional hydroxyl group into its structure which increases the binding affinity. The binding is also affected by many other factors like buffer, interactions such as third hydroxyl and sterics.

1.5 Surface Plasmon Resonance

Surface Plasmon resonance (SPR) technique is an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal. SPR

instrument is used to analyze biomolecular interactions in real time without labeling requirements. The biomolecular interactions which are examined are antigen-antibody, ligand-receptor, protein-nucleic acid interactions, DNA-DNA, DNA-Protein, etc **(17) (18)**. Different surfaces are used for analyzing of different compounds by biacore SPR instruments like, CM5 (carboxymethylateddextran), SA (streptavidin), L1 (Lipophilic dextran), F1 (short dextran) and J1 (unmodified gold surface) **(19)**. CM5 is suitable for routine analysis. J1 is known as user defined-surface for specialized application. In SPR very minutes samples can be used for analysis and the real time results are obtained. SPR forms as a basis for many tools for measurement of adsorption.

In SPR based instruments an optical method is used for the measurement of the refractive index on a flat surface. This flat surface is known as sensor surface and it is used as floor for small flow cell, through which an aqueous solution (running buffer) is passed continuously. The ligand molecule is immobilized on the sensor surface and the analyte is injected as sample through the flow cell under continuous flow. The change in the refractive index is observed as the analyte binds to the ligand. This change in refractive index is measured in real time and results are plotted as response units (RU) versus time (sensorgram). If the running buffer is different from the sample buffer then a response is produced due to the difference in the refractive indices. Then the actual results are obtained by subtracting the response from the sensorgram.

SPR have several advantages like it is versatile to use as any analyte can be detected, providing a biomolecular recognition element which can recognize the analyte. No labels like radioactive or fluorescent labels are required for detection

of the analyte. SPR can perform continuous monitoring as well as one-time analysis. Also the results are obtained in real-time providing potentially rapid responses. SPR also have some limitations like specificity for analyzing the analyte as it depends upon the biomolecular recognition element used and also the sensitivity to interfering effects like nonspecific interactions between sensor surface and sample, background refractive index variations due to different buffers used in running buffer sample, sample temperature etc. **(20)**.

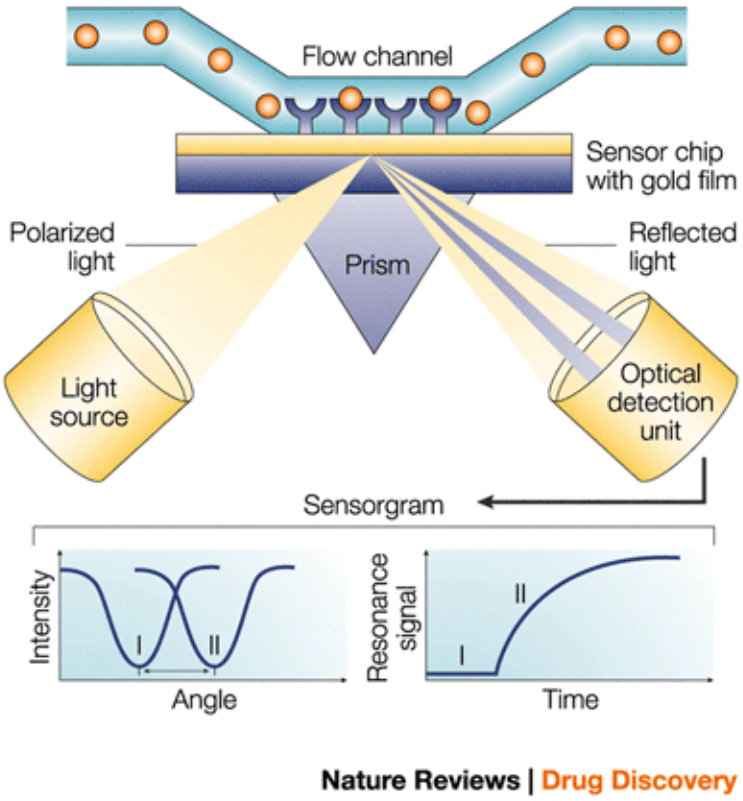


Figure 1.2: SPR detects changes in the refractive index in the immediate vicinity of the surface layer of a sensor chip . The SPR angle shift from I to II in the lower left-hand diagram when molecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored invasively in real time as a plot of resonance signal versus time.

Cited from Nature Reviews Drug Discovery, 1,515-528

CHAPTER II

SYNTHESIS AND CHARACTERIZATION OF BSA-BORONIC ACID CONJUGATES

2.1 Introduction

Boronic acid containing ligands like boronic acid modified nanoparticles **(13, 15,21)**, silica gel **(12, 22, 23)**, peptides **(12)**, magnetic beads **(16),** and polymers **(24-26)** etc. have been explored. These boronic acid ligands have been employed as an artificial carbohydrate receptors **(27)**, sensors, membrane transport agents, and cell surface carbohydrate recognition ligands and as protective agents in synthesis of carbohydrates as well. The broad interest in boronic acid-containing compounds results from their unique interactions with diols: they form cyclic esters with diols in water much more readily than many other acids. In this, study, Bovine Serum Albumin-Boronic Acid conjugates were designed as lectin mimetics for glyco-capturing application, and was synthesized by amidation of carboxylic acid groups in BSA with aminophenyl boronic acid in the presence of EDC/MES (2-(N-morpholino)ethanesulfonic acid), at pH 7.00 overnight, followed by purification on Sephadex G-25 column.

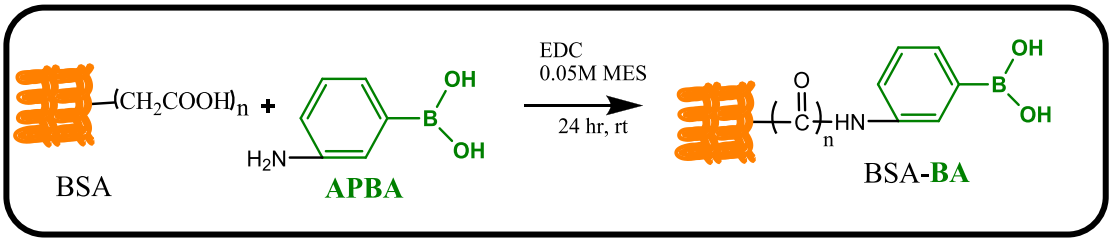


Figure 2.1: Schematic representation of synthesis of BSA-BA conjugates

2.2 Experimental

2.2.1 Materials

Bovine serum albumin, Aminophenylboronic acid, Alizarin Red S and Sugars (Galactose, Glucose, Fructose, Fucose, Mannose, Lactose, Sialic acid, *N*-Acetylglucosamine) were purchased from Aldrich-Sigma Co. Bi-carbonate buffer (pH 8.3) was prepared using 0.1 M $NaHCO_3$ and 0.5 M NaCl, and then adjusted to pH 8.3. PBS buffer (pH 7.4) was prepared using NaCl, KCl, Na_2HPO_4, KH_2PO_4 the adjusted to pH 7.4.

2.2.2 Synthesis of BSA-Boronic acid with conjugates

Bovine serum albumin (100 mg, 1.5 µmol), Aminophenyl boronic acid (20 mg, 130 µmole) were dissolved in 5 mL of 0.05 M MES buffer (2-(*N*-morpholino)ethane sulfonic acid) at pH 6.0. To this mixture 10 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)HCl was added at constant stirring and allow it to react for 2 hrs at room temperature and the pH is adjusted to 7.0 and left overnight at room temperature. The mixture was than subjected to centrifugation in 10,000 MW filters for 10 min to remove the unreacted APBA and also to separate salts and lyophilized. BSA-BA with different densities was prepared using same procedure as above by changing the ratios of BSA to BA.

The obtained products were characterized using Alizarin red assay and SDS-PAGE gel.

2.2.3 Characterization of BSA-Boronic acid conjugates

To 0.5 ml of 1mg/mL solution of BSA-BA in pH 7.4 PBS buffer, 0.5 mL of ARS ($4x10^{-4}$ M) was added and change in the Absorbance was observed by UV-Vis spectroscopy. 0.1 mg/mL solutions of BSA and BSA-BA were prepared and SDS PAGE gel was run to compare the change in molecular weight of the boronic acid modified BSA.

2.2.4 Quantification of BSA-Boronic acid conjugates

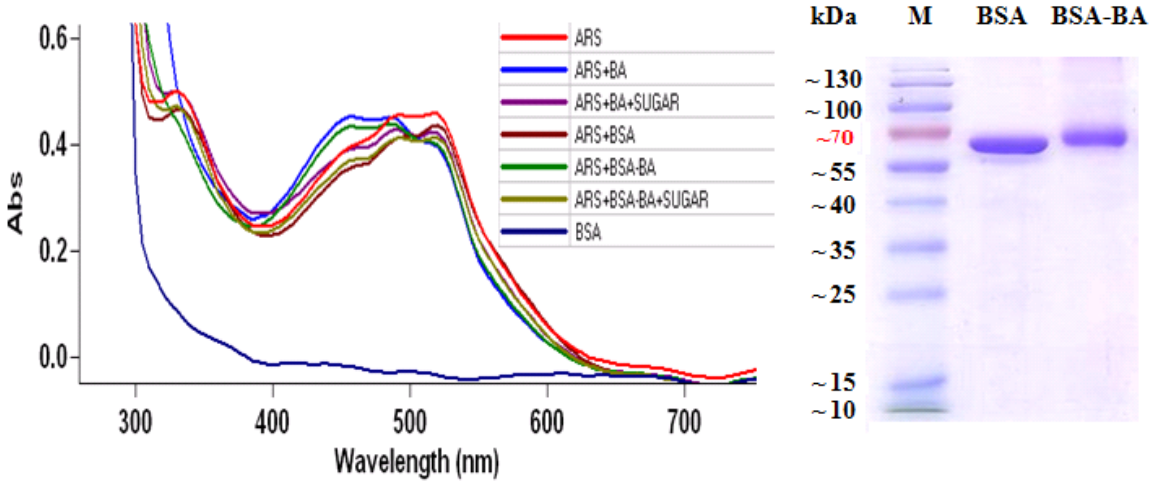
Different concentrations (1 µM, 5 µM) of boronic acid solutions were prepared in pH 7.4 PBS buffer. BSA 1 mg/mL and BSA-BA conjugates each 1 mg/mL were prepared. From these solutions 0.5ml from each concentration was taken and 0.5 ml of $4X10^{-4}$ M ARS solution was added, and then color change is observed. Fluorescence readings were taken and quantified the amount of Boronic acid bound to BSA protein.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization of BSA-BA conjugates

The BSA-BA conjugates which were synthesized were characterized by ARS assay and also SDS PAGE gel electrophoresis. Alizarin Red S (ARS) dye is highly recognized to bind boronic acid with high association constant, ARS when bound to boronic acid a dramatic change in color and fluorescence intensity was

observed **(12,16,24)**. Thus the ARS assay has been used extensively to quantify the boronic acid and also determine the sugar binding affinity to boronic acid ligands by ARS displacement assay **(25)**.ARS showed a color change from pink to yellow when bound to BSA-BA and also shift in wavelength from around 510 nm to 460 nm by UV absorption in PBS (pH 7.4) buffer and then by adding high concentration (1 M) of fructose, the fructose-boronic acid complex is formed to release ARS and the color change from yellow to pink was observed and the shift in wavelength back to 510 nm was observed (Figure 2.2A). Also, the BSA-BA was characterized by SDS-PAGE (Figure 2.2B) where the increase in molecular weight of the BA conjugated BSA was observed significantly.



A) U.V-Vis spectroscopy **B) SDS PAGE gel**

Figure 2.2: Characterization of BSA-BA A. Alizarin Red S assay, B. SDS-PAGE, (M. Marker, 1.BSA, 2. BSA-BA)

2.3.2 Quantification of BSA-BA by ARS assay

The synthesized BSA-BA conjugates were quantified by ARS assay. From the readings taken from fluorescence spectrophotometer the amount of boronic acid

bound to the BSA protein were calculated. Five different densities of BSA-BA were synthesized, quantified by ARS assay monitored through fluorescent spectroscopy (Figure 2.3 A) (Table 2.1) and observed a linear increase in the conjugation of boronic acid to BSA with increase in the concentration of boronic acid to BSA in the reaction (Figure 2.3 B)

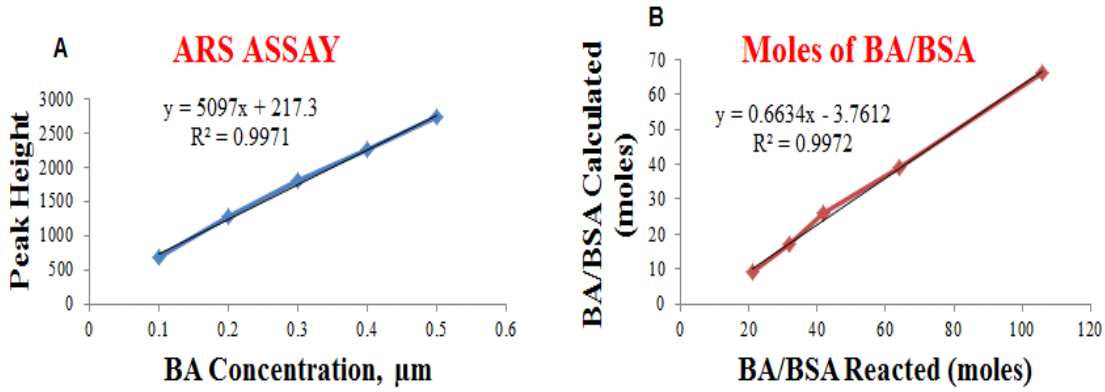


Figure 2.3: A) Calibration curve of ARS by fluorescent spectroscopy B) Increase in the calculated conjugation of APBA to BSA with increase in the reaction concentration ratio of APBA to BSA

Table 2.1 Quantification of BA on BSA by ARS assay

Protein-BA	Protein/APBA[a](moles) (Reaction ratio)	Protein/BA[a](moles) (Product ratio)
BSA-BA 1	1/21	1/9
BSA-BA 2	1/32	1/17
BSA-BA 3	1/42	1/26
BSA-BA 4	1/64	1/39
BSA-BA 5	1/106	1/66

2.3 CONCLUSION

BSA-BA with different densities of boronic acid were synthesized by amidation of boronic acid onto BSA. BSA-BA conjugates were characterized by ARS assay

monitored by UV-Vis Spectroscopy and SDS PAGE gel. Quantification of boronic acid conjugated to BSA was determined by ARS assay monitored by Fluorescence Spectroscopy.

CHAPTER III

BSA-BA MODIFIED SILICA GEL-BASED GLYCO-CAPTURING

3.1 Introduction

Silica gel has been widely used as small, rigid particles for high performance affinity chromatography as it is capable of withstanding high flow rates and /or pressures. Functionalized silica gel for chromatography applications has received vast attention. In this study, BSA-BA was immobilized onto maleimide functionalized silica beads by maleimide-thiol coupling. SB-BSA-BA was characterized by IR spectroscopy. ARS assay was performed with the SB-BSA-BA for quantifying BSA-BA on silica beads while the specific carbohydrate binding capacity of SB-BSA-BA was also evaluated by Alizarin Red S binding assay.

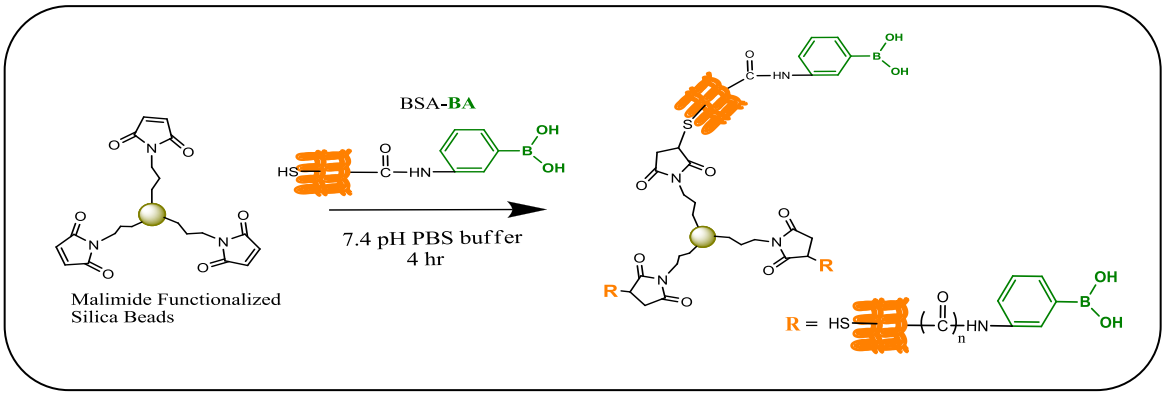


Figure 3.1: Schematic representation of Immobilization of BSA-BA onto silica beads

3.2 Experimental

3.2.1 Immobilization of BSA-BA onto silica beads

45 mg of BSA-BA was dissolved in 3 mL of pH 7.4 PBS buffer, to this mixture 250 mg of maleimide-functionalized silica beads was added and reacted for 4 hrs at room temperature. This mixture was then subjected to centrifugation to remove the unreacted BSA-BA and washed 3 times for 10 min and dried under vacuum overnight. BSA-BA modified silica beads were characterized by IR spectroscopy, for further confirmation. Same experiment was followed for all other BSA-BA2, BSA-BA3, BSA-BA4, BSA-BA5 immobilization, respectively.

3.2.2 Characterization of BSA-BA on silica beads

15 mg of BSA-BA modified silica beads were incubated with ARS ($4X10^{-4}$ M) in pH 7.4 PBS buffer for about 30 min and centrifuged to remove the unreacted ARS solution. This mixture is washed for 3 times with pH 7.4 buffer to remove any unreacted or loosely bound ARS. 1mL of 0.1 M fructose sugar solution was added and reacted for 30 min. Displaced ARS solution was removed by centrifugation and subjected to fluorescence spectroscopy.

3.2.3 Quantification of BSA-BA on silica beads

Different concentrations of ARS solutions were prepared and subjected to UV-Vis spectroscopy to obtain calibration curve. To BSA-BA1-SB, BSA-BA2-SB and BSA-BA3-SB (15 mg) 0.5 ml of $4X10^{-4}$ M ARS solution was added and reacted for 30 min respectively. The mixture was centrifuged and supernatant liquid is

collected and subjected to UV-Vis spectroscopy and quantified the amount of silica beads bound to BSA-BA.

3.2.4 Optimization of BSA-BA on silica beads

15 mg of BSA-BA1, BSA-BA2, BSA-BA3, BSA-BA4, and BSA-BA5 modified silica beads were incubated with ARS ($4X10^{-4}$ M) in pH 7.4 PBS buffer for about 30 min and centrifuged to remove the unreacted ARS solution. These mixtures were washed for 3 times with pH 7.4 buffer to remove any unreacted or loosely bound ARS. 1 mL of 0.1 M fructose sugar solution was added and reacted for 30 min. Displaced ARS solution was removed by centrifugation and subjected to fluorescence spectroscopy.

3.2.5 Optimization of BSA-BA3 on silica beads

Different concentrations of BSA-BA (30 mg-0.4 nmoles, 22 mg-0.3 nmoles, 15 mg-0.2 nmoles, 10 mg-0.1 nmoles, and 5 mg-0.05 nmoles) were immobilized onto 50 mg (0.7 mmoles) of maleimide functionalized silica beads and the reaction is kept for 4 hours at room temperature. The mixture is then subjected to centrifugation to remove unreacted BSA-BA and dried under vacuum. 0.5 mL of $4X10^{-4}$ M ARS solution was added and reacted for 30 min. The bound ARS was displaced by 1 mL of 0.1 M Fructose and displaced ARS was measured by UV-Vis spectroscopy.

3.2.6 Optimization of sugar concentration

To 15 mg of BSA-BA immobilized silica beads 1ml of $4X10^{-4}$M ARS solution was added and reacted for 30 min. This mixture was centrifuged to remove the supernatant liquid. Then add different concentrations (1 M, 0.1 M, 0.01 M, 0.001 M, 0.0001 M, and 0.00001 M) of fructose solution and after 30 min reaction the mixture is subjected to centrifugation. The displaced ARS was subjected to fluorescence spectroscopy.

3.2.7 Sugar binding specificity to BSA-BA-SB at pH 7.4 and pH 8.3

Maleimide functionalized silica beads, BSA modified Silica beads, and BSA-BA modified silica beads each 15 mg were incubated with ARS solution ($4X10^{-4}$M) for 30 min and centrifuged to remove the unreacted ARS solution. This mixture is washed for 3 times with pH 7.4 buffer to remove any unreacted or loosely bound ARS and sugar solutions (Lactose, Fructose, Fucose, Glucose, Galactose, Mannose, Sialic acid, N-acetyl glucosamine) (0.1 M) (1 mL) in pH 7.4 PBS and pH 8.3 $NaHCO_3$ buffer were added and reacted for another 30 min. Displaced ARS solutions were removed by centrifugation and subjected to fluorescence spectroscopy.

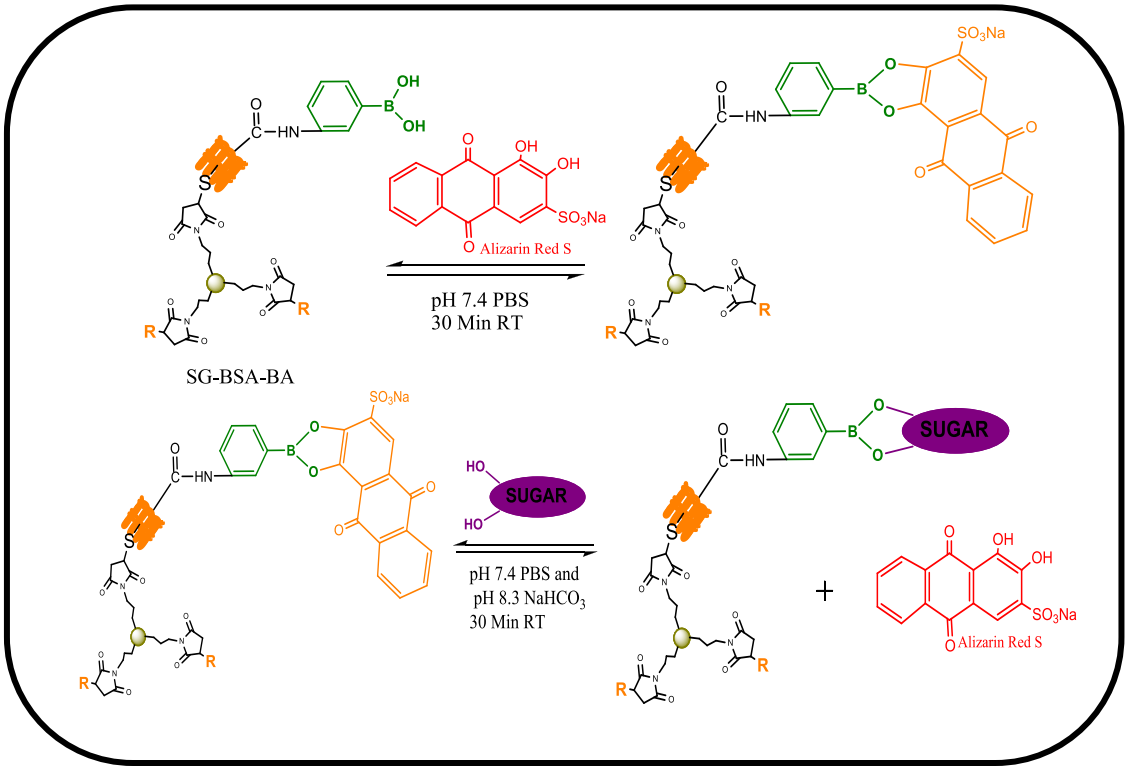


Figure 3.2: Schematic representation of Characterization and sugar binding affinity by ARS assay

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of BSA-BA on silica beads by IR Spectroscopy

BSA-BA immobilized silica beads were characterized by IR spectroscopy. Figure 3.3 shows the spectra of SB, SB-BSA, and SB-BSA-BA. The SB and SA-BSA were taken as standards. The spectra of the SB-BSA-BA were compared with the standard spectra. In SB-Maleimide-BSA-BA spectra the peak from1300-2400 cm^{-1} shows the presence of carbonyl groups from BSA which shows that BSA-BA was immobilized onto the silica beads

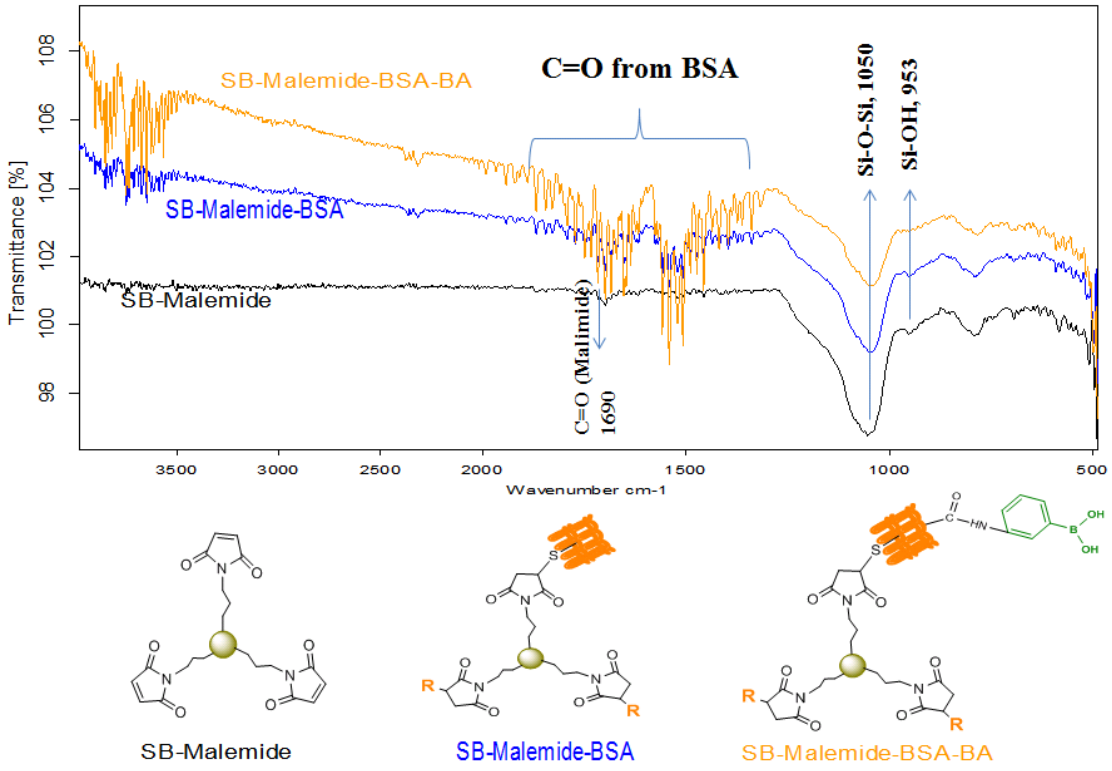


Figure 3.3: IR Spectroscopy of silica beads, BSA modified silica beads and BSA-BA modified silica beads

3.3.2 Characterization of BSA-BA on silica beads by ARS displacement assay

BSA-BA modified silica beads incubated with ARS solution showed brighter color when compared to just Silica gel beads and BSA modified silica beads as shown in Figure 3.4I A1-5 in which the BSA-BA3 modified silica beads displayed high binding of ARS (Figure 3.4I A3) as the BSA-BA3 has higher number of boronic acid moieties when compared to BSA-BA2 , BSA-BA1 (Table 2.1) and also this phenomenon was further confirmed by subjecting the unreacted ARS supernatant (Figure 3.4B1-5) to UV spectroscopy where the unreacted ARS from silica beads (Figure 3.4IC1) , BSA modified Silica beads (Figure 3.4 IC2) showed higher intensity when compared to unreacted ARS from BSA-BA modified silica

beads (Figure 3.4I C3-5). Also when the above ARS bound BSA-BA silica beads were incubated with high concentration of sugar (1M Fructose) in pH 7.4 PBS buffer the ARS was displaced by sugar, it was observed that the brighter color of the silica beads faded (Figure 3.4II A), the solution turned pink due to the displacement of ARS by sugar (Figure 3.4II B) and when the displaced ARS was subjected to fluorescent spectroscopy the BSA-BA3 modified silica beads showed higher intensity (Figure 3.4II C3) when compared to BSA-BA2 modified silica beads (Figure 3.4II C4) and a negligible intensity was observed for BSA-BA1, BSA modified silica beads and just silica beads (Figure 3.4II C1,2,5).

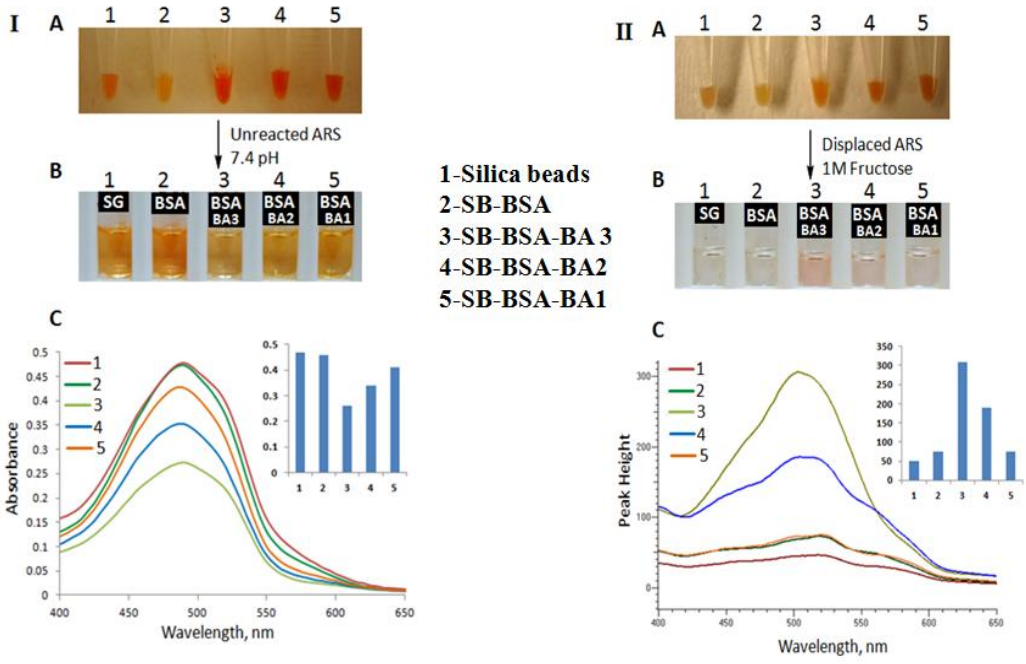


Figure 3.4: ARS displaced assay: 1.Malimide functionalized silica beads 2.BSA modified silica beads, 3. BSA-BA3 modified silica beads, 4. BSA-BA2 modified silica beads, 5. BSA-BA1 modified silica beads, IA: ARS bound to 1, 2, 3, 4, 5. IB: Unbound ARS from 1, 2, 3, 4, 5. IC: UV-Vis spectrum of unbound ARS of IB.IIA: ARS released beads of IA. IIB: ARS displaced from IA. IIC. Fluorescence spectrum of displaced ARS from IA.

3.3.3 Quantification of BSA-BA on silica beads

The BSA-BA immobilized on silica beads were quantified by ARS assay. UV-Vis spectroscopy was taken at 540 nm and a graph (Figure 3.5) was plotted with standards and the amount of the BSA immobilized onto the silica beads was calculated as shown in Table 3.1. For 15 mg of silica beads 0.1 µmoles of BSA was bound. The released ARS was quantified by ARS assay by VU-Vis spectroscopy; this quantified ARS concentration was used to calculate the amount of BSA immobilized on silica beads. As it is known that one mole of ARS reacts with one mole of boronic acid and also the number of moles of boronic acid conjugated to BSA was quantified earlier (Table2.1) , the amount of BSA immobilized on silica beads were quantified to be 6.6 µmoles of BSA per 1 G of Maleimide functionalized silica beads.

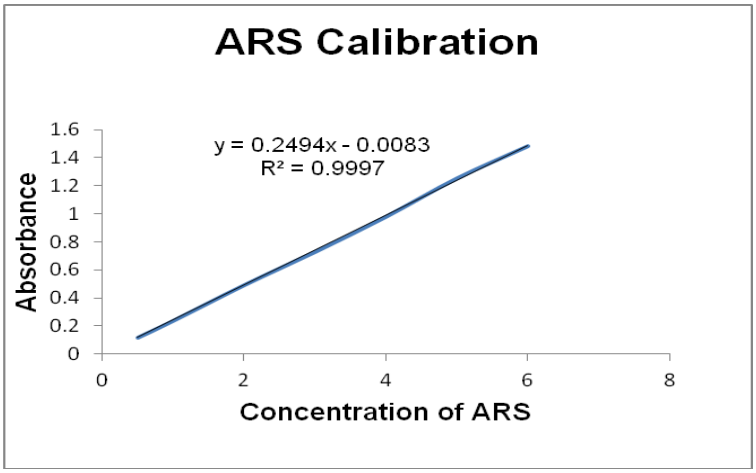


Figure 3.5: Calibration curve of ARS by UV-Vis spectroscopy

Table 3.1 quantification of ARS released from BSA-BA modifies silica beads and quantification of BSA-BA immobilized on silica beads

	µmoles of ARS	µmoles of BA	BA/protein (moles)	BSA on silica beads (15 mg) µmoles	No of µmoles of BSA / 1 g of silica beads
BSA-BA1	2.6	2.6	26	0.1	6.6
BSA-BA2	1.7	1.7	17	0.1	6.6
BSA-BA3	0.96	0.96	9	0.1	6.6

3.3.4 Optimization of BSA-BA on silica beads

All the five BSA-BA (BSA-BA1, BSA-BA2, BSA-BA3, BSA-BA4, BSA-BA5) were tested to select one BSA-BA for further studies, ARS displacement assay was conducted using fructose as a model sugar as earlier and quantified the ARS displaced and BSA-BA3 was chosen for further studies as the BSA-BA 4 and BSA-BA 5 reached plateau (Figure 3.6,Ttable 3.2).

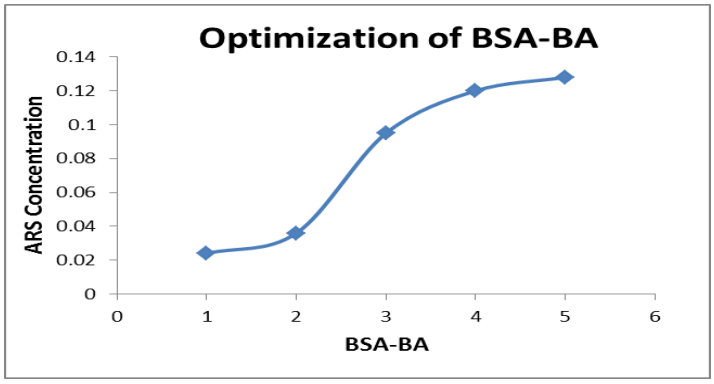


Figure 3.6: optimization of BSA-BA on silica beads

Table 3.2 ARS release from BSA-BA immobilized silica beads, 1. BSA-BA1, 2. BSA-BA2, 3.BSA-BA3, 4.BSA-BA4 and 5. BSA-BA5.

BSA-BA	ARS release (µmoles)
BSA-BA1	0.024
BSA-BA2	0.036
BSA-BA3	0.095
BSA-BA4	0.12
BSA-BA5	0.128

3.3.5 Optimization of BSA-BA3 on silica beads

Before testing the affinities of different sugars to BSA-BA3 modified silica beads, we have optimized the ratios of BSA-BA3 to Malimide functionalized silica beads. Briefly, different ratios of BSA-BA3 ranging from (0.05 to 0.4 nmoles) were incubated with 50 mg of silica beads and tested for the sugar binding affinity following the above ARS displacement assay. The best ratio of BSA-BA to Silica beads was determined to be 0.3 nmoles and used for further studies (Figure 3.6, Table 3.3).

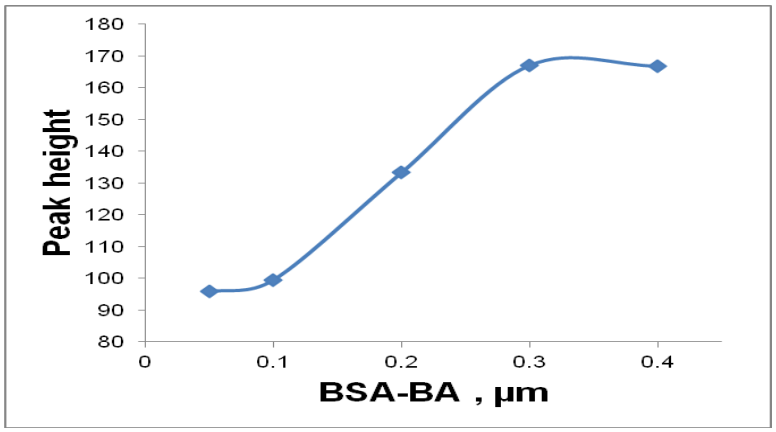


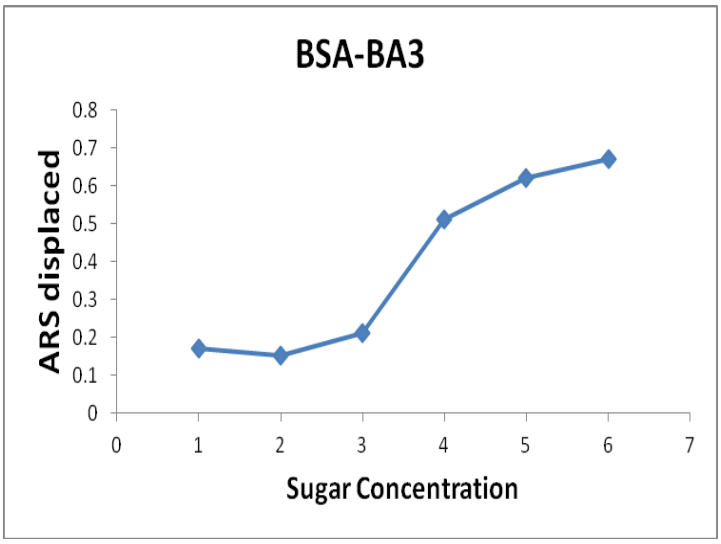
Figure 3.7: optimization of BSA-BA3 onto Silica beads

Table 3.3 optimization of the BSA-BA onto silica beads

Maleimide Silica beads (mg)	BSA-BA (mg)	Peak height (ARS release)
50	5	395
50	10	396
50	15	532
50	22	666
50	30	664

3.3.6 Optimization of sugar concentration

The sugar concentration to displace the ARS was optimized and determined by Fluorescence spectroscopy. Figure 3.8 shows that as the concentration of the sugar increases there is an increase in the binding.



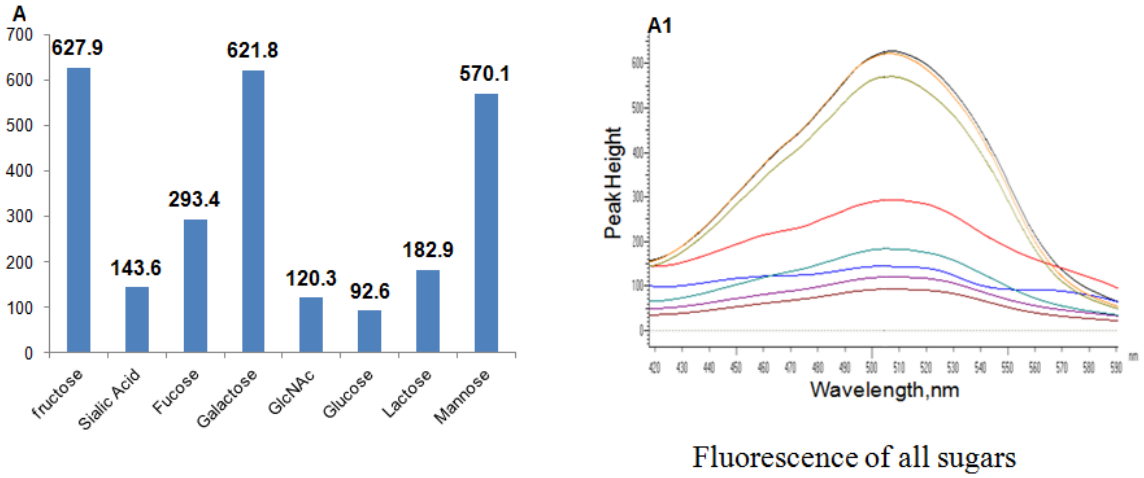
	SUGAR(M)
1	0.00001
2	0.00010
3	0.00100
4	0.01000
5	0.10000
6	1.00000

Figure 3.8: Optimization of sugar concentration

3.3.7 Sugar binding specificity to BSA-BA-SB at pH 7.4 and pH 8.3

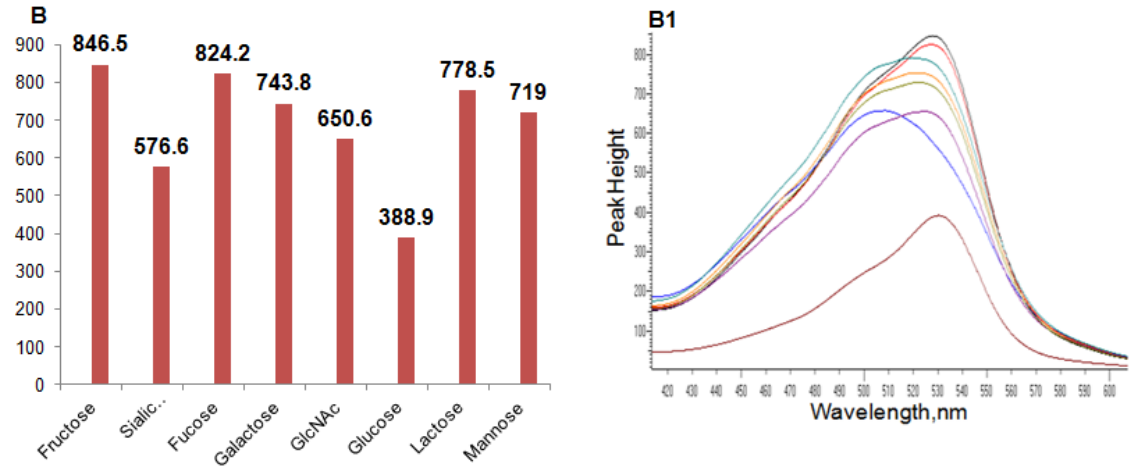
Different sugars have special binding specificities to boronic acid and it is highly pH dependent, we have investigated the specificity of eight common carbohydrates to BSA-BA under two different pH conditions 7.4 and 8.3 by ARS displacement assay. ARS displaced by the sugars was removed and subjected to fluorescence spectroscopy. When the sugars were tested at pH 7.4 PBS buffer, except for fructose, galactose and mannose all other sugars showed very low specificity for BSA-BA (Figure 3.9 A) and even at pH 8.3 fructose, galactose exhibited higher specificity to BSA-BA. Interestingly, sugars that showed low specificity at pH 7.4 displayed higher binding to BSA-BA at pH 8.3 fucose 3 fold, lactose, sialic acid, glucose 4 fold and N-acetylglucosamine 5 fold whereas fructose, galactose and mannose displayed only 1.3 fold increase in binding at 8.3 pH (Figure 3.9B). The sugar binding specificities were shown from higher to

lowest in the graphs at pH 7.4, Fructose > Galactose > Mannose > Fucose > Lactose > Sialic Acid > GlcNAc > Glucose (Figure 3.9A1) and pH 8.3, Fructose > Fucose > Lactose > Galactose > Mannose > GlcNAc > Sialic Acid > Glucose (Figure 3.9B1).



Fluorescence of all sugars

Fructose > Galactose > Mannose > Fucose > Lactose > Sialic Acid > GlcNAc > Glucose



Fluorescence of all sugars

Fructose > Fucose > Lactose > Galactose > Mannose > GlcNAc > Sialic Acid > Glucose

Figure 3.9: Sugar binding specificity to BSA-BA modified silica gel beads A, A1. ARS displaced at pH 7.4; B, B1. ARS displaced at pH 8.3

3.3 CONCLUSION

BSA-BA was immobilized onto maleimide functionalized silica beads by maleimide-thiol coupling. SB-BSA-BA was characterized by IR spectroscopy. ARS assay was performed with the SB-BSA-BA for quantifying BSA-BA on Silica beads while the specific carbohydrate binding capacity of SB-BSA-BA was also evaluated by Alizarin Red S binding assay. It was observed that at pH 8.3 sugars have shown more binding than at pH 7.4, as at higher pH boronic acid gets an additional hydroxyl group which helps to bind more amounts of sugars. In our results, the interaction between the sugars and SB-BSA-BA was in the order Fructose > Galactose > Mannose > Fucose > Lactose > Sialic Acid > GlcNAc > Glucose at pH 7.4 and Fructose > Fucose > Lactose > Galactose > Mannose > GlcNAc > Sialic Acid > Glucose at pH 8.3. It is advantage to use the SB-BSA-BA for enrichment and separation of carbohydrates. By optimizing the BSA-BA, a specific glyco-capturing system will be obtained.

CHAPTER VI

SPR ANALYSIS OF BSA-BA BASED GLYCO-CAPTURING

4.1 Introduction

Surface Plasmon Resonance (SPR) Analysis of sugars binding has been used widely. In this study, the interaction of sugars with immobilized BSA-BA was also investigated with SPR (BI 2000, Biosensing Instrument). The major advantages of SPR assay are that is a label free assay and monitors the binding in real time. Initially, BSA-BA was immobilized on gold sensor chip and the sugar binding affinities were tested at both pH 7.4 and pH 8.3.

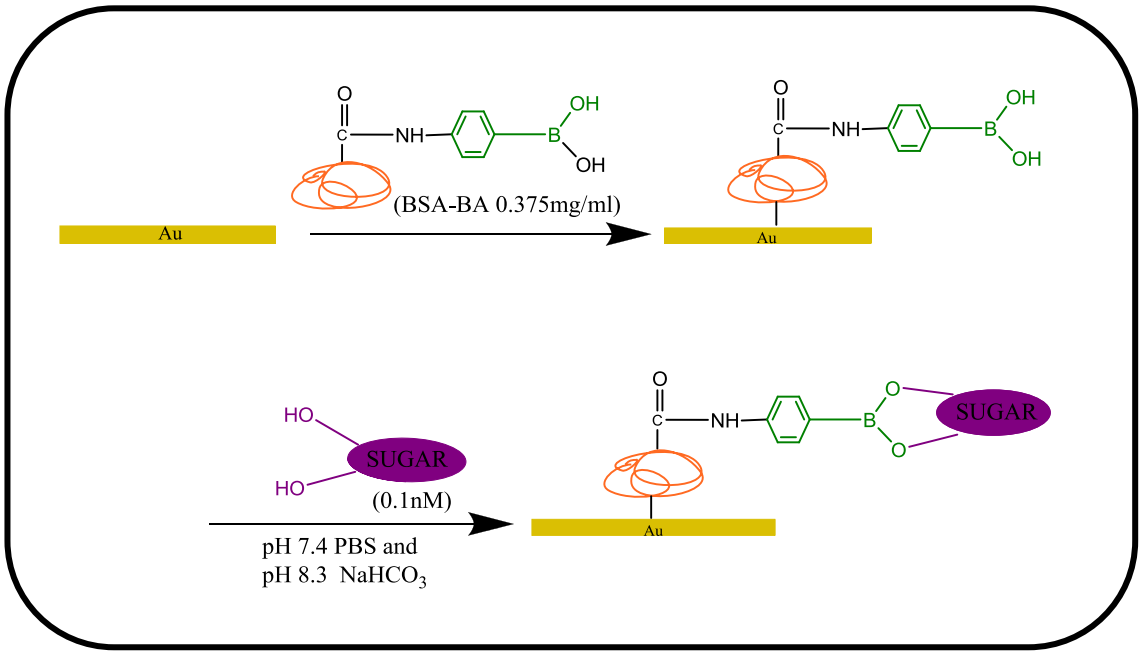


Figure 4.1: Schematic representation of SPR analysis for sugar binding studies

4.2 Experimental

4.2.1 BSA-BA3 immobilization on gold chip

0.375 mg/ml BSA-BA3 solution was prepared in pH 7.0 solution. A fresh gold chip was taken molded on SPR instrument. pH 7.0 solution was used as running buffer and 100 µL of prepared BSA-BA solution was injected at a flow rate of 7 µL/Min for about 768 seconds.

4.2.2 Sugar binding to BSA-BA in pH 7.4

Sugar solutions of 0.1 nM concentration were prepared in pH 7.4 PBS buffer. Same buffer is used as running buffer and base line is allowed to stabilize. 60 µL of each sugar solution was then injected into the channels at a flow rate of 15 µL/Min for about 192 seconds onto the BSA-BA immobilized gold chip. The steady state responses were taken for all the sugars from 0 to 375 seconds i.e., from association phase until the start of dissociation phase.

4.2.3 Sugar binding to BSA-BA in pH 8.3

Sugar solutions of 0.1 nM concentration were prepared in pH 8.3 $NaHCO_3$ buffer. Same buffer is used as running buffer and base line is allowed to stabilize. 60 µL of each sugar solution was then injected into the channels at a flow rate of 15 µL/Min for about 192 seconds onto the BSA-BA immobilized gold chip. The steady state responses were taken for all the sugars from 0 to 375 seconds i.e., from association phase until the start of dissociation phase.

4.2.4 Glycopolymers binding to BSA-BA in pH 8.3

2-6' sialyl lactose monomer and polymer, 2-3' sialyl lactose monomer and polymer, and lactose polymer solutions of 0.1 nM concentration were prepared in pH 8.3 $NaHCO_3$ buffer. Same buffer solution is used as running buffer allowed the base line to stabilize. Then 60 µL of each glycopolymers was injected at a flow rate of 15 µL/Min for about 192 seconds. The steady state responses were taken for all the sugars from 3 to 375 seconds i.e., from association phase until the dissociation phase. Figure 4.1 shows the schematic representation of the procedure for SPR analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 SPR Analysis for sugar binding studies

For further confirmation of sugar binding to BSA-BA, surface Plasmon resonance (SPR) technique was employed, BSA-BA was immobilized on the gold chip *via* thiol–Au coupling in pH 7.0 for 10 min at 7 µL/Min (Figure 4.2). Followed by injecting the sugar solution (0.1 µM) at flow rate of 15 µL/Min for 3 min in both pH 7.4 PBS buffer (Figure 4.3 A) and pH 8.3 $NaHCO_3$ buffer for studying sugar binding specificities (Figure 4.3 B). All the sugars exhibited approximately similar binding response between 30 RU to 40 RU (Figure 4.3A1) at pH 7.4, whereas the binding responses of all the sugars were increased to around 60 RU (Figure 4.3B1) at pH 8.3. Though the binding specificities of all the sugars were not similar to the ARS assay the binding affinity of the sugars were higher at pH 8.3 by SPR which was also observed by ARS assay. Also the association constant of almost all the sugars was observed to be higher at pH 8.3 than at pH 7.4 and the

dissociation of all the sugars except lactose was observed to be almost 2 fold lower at pH 8.3 than at pH 7.4 (Table 4.1) indicating that the binding much stronger at pH 8.3 than at pH 7.4.

Further we have also tested the binding affinity of glycopolymers that were previously synthesized, lactose polymer **(28)**, 23 sialyl lactose polymer and 26 sialyl lactose polymer **(29)** were tested at pH 8.3 and observed that the binding response was higher for polymers when compared to respective monomers.

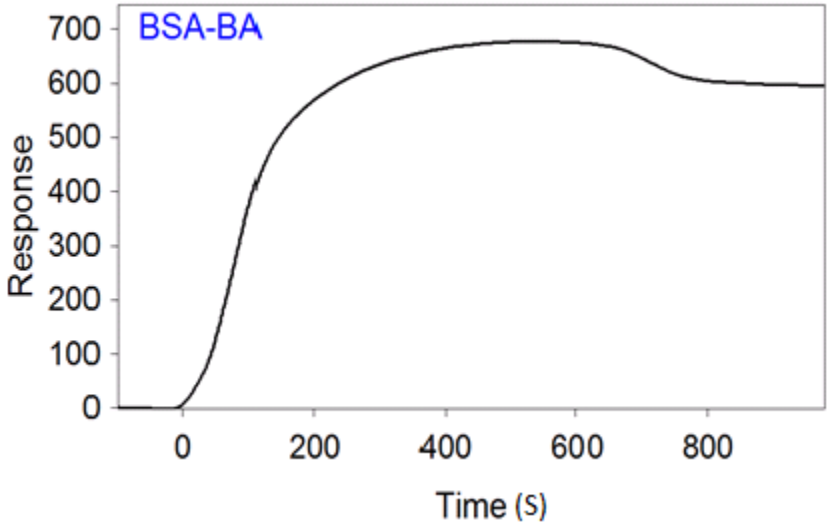


Figure 4.2: SPR sensorgram of BSA-BA3 immobilization on gold chip in pH 7.0 for 10 min at 7 µL/Min

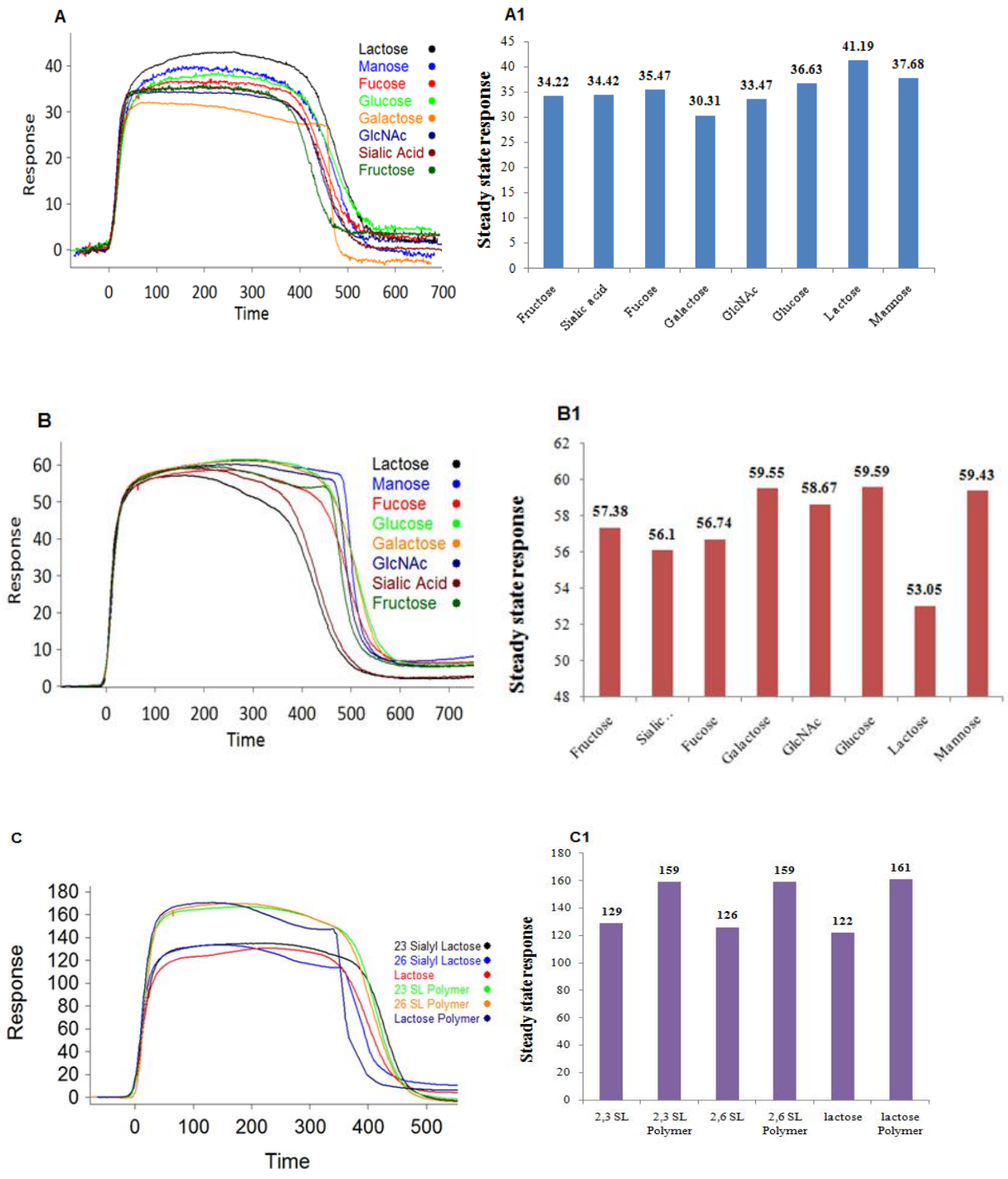


Figure 4.3: SPR Sensorgrams of sugars binding to BSA-BA A. Sugars binding to BSA-BA at pH 7.4; A1. Steady state response of sugars binding to BSA-BA at pH 7.4; B. Sugars binding to BSA-BA at pH 8.3; B1. Steady state response of sugars binding to BSA-BA at pH 8.3; C. Glycopolymers and monomers binding to BSA-BA at pH 8.3; C1 Steady state response of sugars binding to BSA-BA at pH 8.3

Table 4.1 k_a, k_d values of all sugars

	7.4 pH			8.3 pH		
	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (M)	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (M)
Lactose	2.22×10^{5}	8.91×10^{-3}	40.07×10^{-8}	6.27×10^{5}	1.65×10^{-2}	26.37×10^{-8}
Mannose	2.09×10^{5}	1.11×10^{-2}	53.29×10^{-8}	3.81×10^{5}	5.63×10^{-3}	14.78×10^{-8}
Fucose	2.63×10^{5}	1.06×10^{-2}	40.40×10^{-8}	4.48×10^{5}	6.71×10^{-3}	14.99×10^{-8}
Glucose	2.27×10^{5}	8.34×10^{-3}	36.74×10^{-8}	3.62×10^{5}	6.11×10^{-3}	16.88×10^{-8}
Galactose	3.25×10^{5}	1.29×10^{-2}	39.73×10^{-8}	3.78×10^{5}	6.30×10^{-3}	16.66×10^{-8}
GlcNAc	4.33×10^{5}	1.21×10^{-2}	28.10×10^{-8}	4.07×10^{5}	6.75×10^{-3}	16.57×10^{-8}
Sialic Acid	3.79×10^{5}	1.28×10^{-2}	33.85×10^{-8}	4.75×10^{5}	1.44×10^{-2}	30.41×10^{-8}
Fructose	3.87×10^{5}	1.85×10^{-2}	47.87×10^{-8}	4.05×10^{5}	7.52×10^{-3}	18.56×10^{-8}

4.4 CONCLUSION

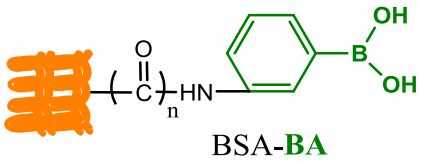
SPR analysis was conducted to study the sugar binding affinity of the BSA-BA. BSA-BA was immobilized onto the gold chip and then sugar binding affinity of the BSA-BA was tested. The results were evaluated using the software. From this experiment we conclude that as like in ARS assay, sugar binding towards BSA-BA was seen more at pH 8.3 rather than at pH 7.4. In our results, the interactions between the sugars and BSA-BA on the gold chip were in the order Lactose > Mannose > Glucose > Fucose > Sialic acid > Fructose > GlcNAc > Galactose at pH 7.4 and Glucose > Galactose > Mannose > GlcNAc > Fructose > Fucose > Sialic acid > Lactose at pH 8.3 and glycopolymers shows more binding that the monomers.

CHAPTER V

SUMMARY

Lectin mimetic boronic acid macromolecules (BSA-BA) were synthesized by conjugation of aminophenyl boronic acid to bovine serum albumin. BSA-BA with different densities of boronic acid were synthesized by amidation of boronic acid onto BSA and were characterized by ARS assay monitored by UV-Vis Spectroscopy and SDS PAGE gel. Quantification of boronic acid conjugated to BSA was determined by ARS assay monitored by Fluorescence Spectroscopy. The boronic acid conjugates were immobilized onto maleimide functionalized silica beads *via* thiol-maleimide coupling. SB-BSA-BA was characterized by IR spectroscopy. ARS assay was performed with the SB-BSA-BA for quantifying BSA-BA on silica beads while the specific carbohydrate binding capacity of SB-BSA-BA was also evaluated by Alizarin Red S binding assay. It is seen that at pH 8.3 sugars have shown more binding than at pH 7.4. Further SPR analysis was conducted to study the sugar binding affinity of the BSA-BA. BSA-BA was immobilized on the gold chip *via* thiol-Au coupling and then sugar binding affinity of the BSA-BA was tested. The specificity of different carbohydrates to immobilized BSA-BA was investigated at different pH conditions, proving that the carbohydrate specificity to boronic acid is pH dependent. These lectin mimetics

will provide an important tool for glycomics and glycoproteomics research and applications.



BSA-**BA**

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